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Establishment of the cells useful for murine interleukin-18 bioassay by introducing murine interleukin-18 receptor cDNA into human myelomonocytic KG-1 cells

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Abstract

We genetically engineered human myelomonocytic KG-1 cells by introducing cDNA of murine interleukin-18 receptor (MuIL-18R) and established human cells which were capable of responding to MuIL-18. These cells expressed larger number of MuIL-18R (> 13,000 sites/cell) than intrinsic human IL-18 receptor (HuIL-18R) (< 2,500 sites/cell). And the cells responded to MuIL-18 as well as to HuIL-18 in a dose-dependent manner, and produced large amounts of interferon-γ (IFN-γ). We could estimate the amount of murine IL-18 based on the amounts of IFN-γ produced by these cells. The stoichiometry was observed up to 150 ng/ml of MuIL-18. By using these cells, a large amount of MuIL-18 (448 ± 89.2 ng/ml) was detected in sera of *Propionibacterium acnes* (*P. acnes*)/lipopolysaccharide (LPS)-treated endotoxic mice (the same conditions in which IL-18 was first identified). These cells provide us with a useful tool for determining the bioactivity of MuIL-18.

Keywords: Cytokine; Bioassay; Murine IL-18; Human cells; Recombinant DNA

1. Introduction

Interleukin-18 (IL-18) is a cytokine, which has pleiotropic effects mainly on immune cells (Okamura et al., 1995; Ushio et al., 1996; Kohno and Kurimoto, 1998). So far, augmentation of production of T helper type 1 (Th1)-type cytokines, interferon-γ (IFN-γ), granulocyte/macrophage colony stimulating factor (GM-CSF), and interleukin-2 (IL-2) by T cells; enhancement of Fas ligand expression by Th1 cells; enhancement of the cytotoxicity of natural killer (NK) cells, and also anti-tumor effects were reported (Kohno et al., 1997; Dao et al., 1996; Ohtsuki et al., 1997; Tsutsui et al., 1996; Micallef et al., 1996, 1997a,b). Based on its activities, IL-18 has been gathering great interests from investigators.

To better understand the mechanisms of action, we have been studying the signaling pathways of IL-18, and reported involvement of Lck/MAPK ki-

Abbreviations: IL-18, interleukin-18; IFNγ, interferon-γ; IL-18R, interleukin-18 receptor; cDNA, complementary DNA

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nase pathway and IRAK/TRAF6/NF-κB pathway, which was crucial for IFN-γ and IL-2 production by IL-18 (Matsumoto et al., 1997; Tsuji-Takayama et al., 1997; Kojima et al., 1998). Recently, we further purified and identified a component of IL-18 receptor (IL-18R) (Torigoe et al., 1997). We also determined the partial amino acid sequences, which were included in the sequence of an orphan receptor reported as IL-1 receptor-related protein (IL-1Rrp) (Parnet et al., 1996), and cloned the cDNA. The IL-18R itself was able to bind IL-18 and to transduce the signals toward activation of NF-κB in transient expression experiments.

To study the physiological/pathological roles and biological effects of a certain substance, mouse models have been used often. The in vivo effects of IL-18, such as enhancement of the cytotoxicity of NK cells and anti-tumor effects were reported in mouse experimental models (Micallef et al., 1997a,b). In such models, it is very important to trace the amount of IL-18. For bioassay of murine IL-18 (MuIL-18), however, determining the amount of IFN-γ produced by mouse splenocytes is the only way (Okamura et al., 1995). This system is useful but very laborious and less stable. A More convenient and stable bioassay system is required. In the previous report, we established a convenient bioassay system for human IL-18 (HuIL-18) by using human myelomonocytic KG-1 cells (Konishi et al., 1997). In this paper, we report the establishment of genetically engineered human cells useful for bioassay of MuIL-18.

2. Materials and methods

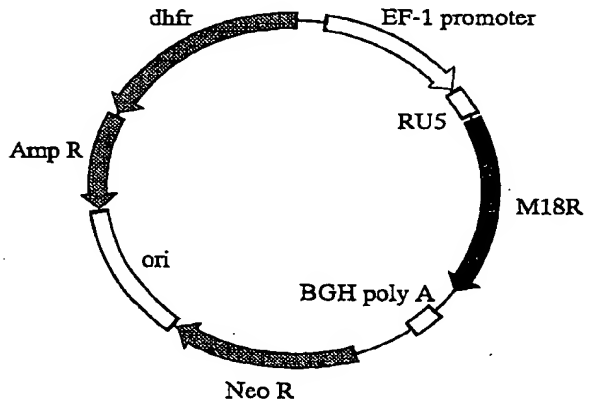
2.1. Cells, reagents, and cytokines

Human myelomonocytic KG-1 cells (ATCC CCL246) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin under an atmosphere of 5% CO_2/95% air at 37°C.

Geneticin (G-418) was purchased from Gibco BRL (Rockville, MD). HuIL-18 and MuIL-18 were prepared by expression of the corresponding cDNA in *Escherichia coli* and purified to homogeneity as described (Okamura et al., 1995; Ushio et al., 1996).

2.2. Construction of an expression vector for MuIL-18R cDNA

According to the reported nucleotide sequence of MuIL-18R cDNA (MuIL-1 Rrp, GenBank accession number U43673) (Parnet et al., 1996), oligonucleotide primers (5'AGAGGAACCACCCACAACGATCCT3' and 5'TGAATAGGCACACGCATGACCTCT3') were designed and synthesized. MuIL-18R cDNA (1.7 kbp) was amplified from murine liver RNA by reverse transcription-polymerase chain reaction (RT-PCR) using Pfu DNA polymerase (STRATAGENE, La Jolla, CA) (95°C, 45 s; 72°C, 3.5 min; 10 cycles and 95°C, 45 s; 68°C, 3.5 min; 35 cycles), cloned into pCRScript Cam SK(+) (STRATAGENE), and the sequence was ascertained. A eukaryotic expression vector, pREF-XN, was constructed with the EF-1 promoter of pEF-BOS vector (Mizushima and Nagata, 1990), the dihydrofolate reductase unit of pSV2dhfr (ATCC 37146), and the backbone of pRc/CMV vector (Invitrogen, San Diego, CA). The cDNA of IL-18R was ligated into *XhoI*/*NotI* sites of the vector and the resulting MuIL-18R expression vector was designated pRcEFM18R (Fig. 1).



pRcEFM18R

Fig. 1. Structure of a MuIL-18R expression vector, pRcEFM18R. M18R murine IL-18 receptor cDNA, EF-1 promoter human elongation factor-1 (EF-1) promoter, *AmpR*; ampicillin resistant gene, *NeoR*; neomycin resistant gene, dhfr; murine dihydrofolate reductase expression cassette, RU5; adenovirus RU5 sequence, BGH polyA; poly adenylation signal of bovine growth hormone, ori; replication origin.

2.3. Transfection of the MuIL-18R expression vector into human KG-1 cells and cloning of the transformed cells

KG-1 cells were washed twice with RPMI-1640 medium and 1×10^7 cells were used for transfection. The cells were transfected with 50 μg of the MuIL-18R expression vector, pRcEFM18R, by Electroporation (260 V 960 μF; Gene Pulser; Bio Rad Labs., Hercules, CA). The transformed cells were selected in the presence of 400 μg/ml G-418 and cloned.

2.4. Receptor binding analyses

Receptor binding of ^{125}I-labeled MuIL-18 or HuIL-18 was examined on the cells as reported. Briefly, 2×10^6 cells were suspended in RPMI-1640 containing 0.1% NaN_3 and 100 mM HEPES, pH 7.2 and incubated for 1 h at 4°C with ^{125}I-labeled MuIL-18 or HuIL-18 (~4 ng). The unbound IL-18 was separated from the cells, and the cell-bound ^{125}I count was determined. To determine the specific binding of IL-18, non-specific binding was determined in the presence of 3 μg unlabeled cognate ligand and subtracted it from the total binding. For more detailed analyses, Scatchard analyses were carried out.

2.5. Preparation of mouse serum samples

To prepare mouse serum containing endogenous MuIL-18, the endotoxic shock was induced in C57BL/6 mice (Charles River Japan, Yokohama, Japan) as described (Okamura et al., 1995). Briefly, the mice were treated with 500 μg of heat-killed *Propionibacterium acnes* (*P. acnes*) for 1 week and challenged with 1 μg of lipopolysaccharide (LPS). Two hours after the LPS challenge, blood samples were taken from the heart under proper anesthetization and sera were prepared. All animal procedures described were approved by the guidance committee for animal experiments at our institute.

2.6. Responsiveness of the cells for IL-18

Responsiveness of the cells for IL-18 was examined based on the IFN-γ production. The MuIL-18R-expressing KG-1 cells were washed and resuspended at a concentration of 5×10^5 cells/ml with RPMI-1640 medium. After 2 days of preculture, the cell concentration was adjusted again to 1×10^6 cells/ml with RPMI-1640 containing 10% FCS and the indicated amounts of MuIL-18, HuIL-18, or serum sample were added to the cells. One day later, the culture supernatants were recovered and the amounts of IFN-γ produced were determined by a sandwich enzyme-linked immunosolvent assay (ELISA) as described (Konishi et al., 1997). Briefly, the culture supernatant was incubated with immobilized mouse anti-human IFN-γ monoclonal antibody, mAb-IFN-γ-15. The bound IFN-γ was further incubated with horse radish peroxidase (HRPO)-conjugated another mouse anti-human IFN-γ monoclonal antibody, mAb-IFN-γ-6 and detected with hydrogen peroxide and *o*-phenylenediamine.

3. Results

3.1. Establishment of MuIL-18R-expressing human myelomonocytic KG-1 cells

It is commonly accepted that the ligand–receptor interaction determines the species specificity of the ligand action. We expected that introducing MuIL-18R into proper human cells provides the responsiveness to MuIL-18. By introducing an expression vector, pRcEFM18R (Fig. 1), into human myelomonocytic KG-1 cells, we established transformed clones that express MuIL-18R. As expected, the transformants responded to MuIL-18 and produced HuIFN-γ. Based on the results of preliminary experiments (data not shown), two clones, KG/M18R3 and KG/M18R20, were chosen.

3.2. Binding analyses of HuIL-18 or MuIL-18 on KG/M18R3 and KG/M18R20

We determined the characteristics of endogenous HuIL-18R and exogenous MuIL-18R expressed on the surface of KG/M18R3 and KG/M18R20 clones. As shown in Table 1, the parental KG-1 cells possessed 2,500 binding sites with 3.47 nM of K_d for HuIL-18. The binding of MuIL-18 to KG-1 cells was also detected, but the specific binding was only

Table 1
Scatchard analyses of HuIL-18 and MuIL-18 bindings on MuIL-18R-expressing KG-1 clones

	HuIL-18 binding		MuIL-18 binding	
	Sites/cell	K_d (nM)	Sites/cell	K_d (nM)
KG-1	2,500	3.47	U.C.	U.C.
KG/M18R3	667	1.23	14,667	0.33
KG/M18R20	1,333	2.47	13,333	0.71

U.C.; detected but unable to be calculated.
The binding characteristics of the cells were determined by Scatchard analyses using 2×10^6 cells and either HuIL-18 or MuIL-18 labeled with ^{125}I.
The values are averages of triplicate experiments.

detectable and it was not possible to calculate the number of sites and the K_d value. As we reported before, human KG-1 cells were capable to respond to MuIL-18 in approximately 100-fold less sensitivity (Konishi et al., 1997). The results of binding analyses seemed to reflect the weak cross-reactivity between HuIL-18 and MuIL-18.

The transformed clones, both KG/M18R3 and KG/M18R20, expressed a large number of MuIL-18R (14,667 and 13,333 sites) with semi-nano molar K_d value for MuIL-18 (0.33 and 0.71 nM). These clones still possessed the binding capacity for HuIL-18, 667 sites/cell with 1.23 nM in KG/M18R3 and 1,333 sites/cell with 2.47 nM in KG/M18R20. In the following experiments, we used KG/M18R20 cells as a representative clone.

3.3. Responsiveness of the transformed cells expressing MuIL-18R to HuIL-18 or MuIL-18

Next we examined the responsiveness of KG/M18R20 cells to HuIL-18 or MuIL-18 using IFN-γ production as an index. The cells were stimulated with 0–300 ng/ml of either HuIL-18 or MuIL-18 for 1 day and the amounts of IFN-γ produced in the culture supernatant were determined by ELISA. As shown in Fig. 2, the cells produced IFN-γ upon stimulation with MuIL-18 as well as HuIL-18 in a dose dependent manner. And the dose–response curve of MuIL-18 was similar with that of HuIL-18, but a little deeper. The stoichiometry was observed up to 150 ng/ml, similar with HuIL-18 and MuIL-18.

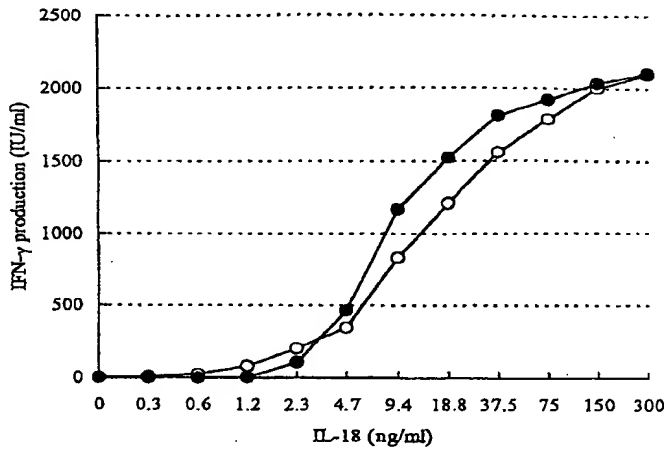


Fig. 2. IFN-γ production by KG/M18R20 cells stimulated with Hu or MuIL-18. KG/M18R20 cells were pretreated with serum free medium for 2 days and stimulated with the indicated amounts of either HuIL-18 or MuIL-18 for 1 day. The amounts of IFN-γ produced in the supernatants were determined by ELISA. Two independent experiments were carried out. And the data are averages of a representative duplicated experiment. ●; MuIL-18, ○; HuIL-18.

3.4. Detection of IL-18 in mouse serum with endotoxic shock

To evaluate the usefulness of KG/M18R20 cells for determining MuIL-18, we examined the amount of IL-18 in mouse serum with endotoxic shock by *P. acnes*/LPS. As shown in Fig. 3, sera of endotoxic

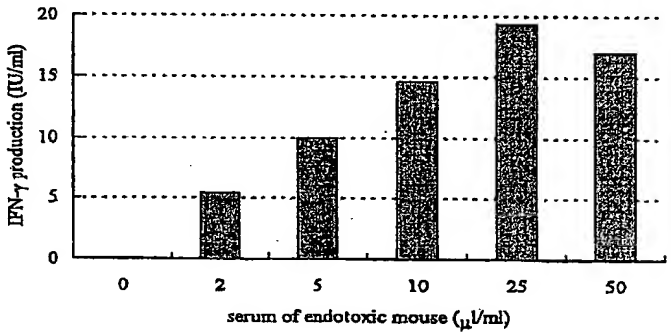


Fig. 3. IFN-γ production by KG/M18R20 cells stimulated with serum of mouse with endotoxic shock. KG/M18R20 cells were pretreated as described in Fig. 2 and stimulated with the indicated amounts of serum of the *P. acnes*/LPS-treated mice for 1 day. The amounts of IFN-γ produced in the supernatants were determined by ELISA. Two independent experiments were carried out. The values are averages ($n = 5$) from a representative experiment.

Table 2
Amount of MuIL-18 in mouse serum determined with KG/M18R20 cells

MuIL-18 in mouse serum (ng/ml)	
Control	*P. acnes*/LPS-treated
N.D.	448 ± 89.2

N.D.; not detected.
The amount of MuIL-18 was estimated based on the IFN-γ production by KG/M18R20 cells stimulated with serum, referring to Fig. 2.
The value is an average ± SD ($n = 5$).

mice stimulated KG/M18R20 cells and led them to produce IFN-γ in a dose dependent manner. But a large amount of serum (more than 5%) showed a suppressive effect on the IFN-γ production by KG/M18R20 cells. On the other hand, the control serum did not have a stimulatory effect. Based on the amounts of IFN-γ produced, the amount of MuIL-18 in the sera of mice with endotoxic shock was determined to be 448 ± 89.2 ng/ml (Table 2).

4. Discussion

We established genetically engineered human cells which are capable of responding to MuIL-18 and producing HuIFN-γ. Introducing an EF-1 promoter-drived MuIL-18R expression vector into human myelomonocytic KG-1 cells provided us with transformed human cells which express a large number of MuIL-18R on their surface. The expression level was more than 5-fold of intrinsic HuIL-18R (13,333 vs. 2,500 sites/cell). The EF-1 promoter is one of the most powerful promoters in mammalian cells (Mizushima and Nagata, 1990) and this was also the case in KG-1 cells.

By expressing MuIL-18R, KG-1 cells became capable of responding to MuIL-18, suggesting that the receptor determined the species specificity. The intrinsic responsiveness to HuIL-18 was not affected by the genetic engineering, and the cells produced IFN-γ as did the parental cells (Konishi et al., 1997). These results suggest that the MuIL-18R we used is responsible for biological actions of IL-18, at least, production of IFN-γ and that, if any, other signaling molecules in the IL-18 actions do not bear the species specificity. The further studies on the signaling molecules are in progress.

A representative clone, KG/M18R20 produced IFN-γ upon stimulation with MuIL-18 in a dose dependent manner. The stoichiometry was observed up to 150 ng/ml of MuIL-18. The responsiveness was similar to that for HuIL-18. As mentioned above, however, the number of MuIL-18R expressed was much higher (13,333 sites/cell) than that of HuIL-18R, (1,333 sites/cell). These results suggest that the number of receptor does not determine the sensitivity to the ligand.

Referring the dose–response curve of the KG/M18R20 cells, we could determine the amount of bioactive MuIL-18. So, we examined the sera of mice treated with *P. acnes*/LPS. These were the same conditions in which IL-18 was first identified. The amount of MuIL-18 in the sera of the endotoxic mice was determined extremely high, 448 ± 89.2 ng/ml based on the IFN-γ production.

An advantage of the cells we used is that they are of human-origin. Many cytokines have the strict species specificity and it is generally difficult for cytokines to exert the effects on the cells of different species. In the case of determining the amount of MuIL-18, we suppose that there would be less chance for the KG/M18R20 cells to be affected by other cytokines. In addition, the cells did not respond to IL-12, a well-known potent inducer of IFN-γ (data not shown). However, we need to ascertain this issue.

Taken together, these genetically engineered human cells which express MuIL-18R seem very useful to trace the amount of bioactive MuIL-18. And they provide us with a simple bioassay system for MuIL-18.

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